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Optimization of Enzymatic Gas-Phase Reactions by Increasing the Long-Term Stability of the Catalyst

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Enzymatic gas-phase reactions are usually performed in continuous reactors, and thus very stable and active catalysts are required to perform such transformations on cost-effective levels. The present work is concerned with the reduction of gaseous acetophenone to enantiomerically pure (*R*)-1-phenylethanol catalyzed by solid alcohol dehydrogenase from *Lactobacillus brevis* (LBADH), immobilized onto glass beads. Initially, the catalyst preparation displayed a half-life of 1 day under reaction conditions at 40 °C and at a water activity of 0.5. It was shown that the observed decrease in activity is due to a degradation of the enzyme itself (LBADH) and not of the co-immobilized cofactor NADP. By the addition of sucrose to the cell extract before immobilization of the enzyme, the half-life of the catalyst preparation (at 40 °C) was increased 40 times. The stabilized catalyst preparation was employed in a continuous gas-phase reactor at different temperatures (25–60 °C). At 50 °C, a space–time yield of 107 g/L/d was achieved within the first 80 h of continuous reaction.

Introduction

Enzymatic gas/solid catalysis is a particular example of biotransformation in a nonconventional environment. Its characteristics combine common aspects of other nonconventional systems: employment of quasi-dry enzymes immobilized onto inert supports, reduced and controlled presence of water in the system, and the ability to convert poorly water-soluble substrates (1). A peculiar aspect of enzymatic gas/solid catalysis is that substrates react in gaseous form, i.e., there is no fluid solvent in the system. This is the most significant advantage but at the same time also the most important limitation of enzymatic gas/solid catalysis. Without solvent, the risk of degradation of the enzyme is reduced and product recovery is extremely simplified (2). On the other hand, substrate concentrations within the system are usually low and only volatile compounds, such as flavors or pheromones, can be employed with success.

To profit from the advantages offered by enzymatic gas/solid catalysis and to partially overcome its outlined limitations, high reaction temperatures and continuously operated systems with extended catalyst lifetimes are of great importance, i.e., the employed enzyme has to exhibit a high thermostability. Up to now, enzymatic gas-phase reactions were mostly conducted with stable enzymes, such as alcohol oxidases (3–7), cutinases (2, 8–11), and lipases (2, 8, 11–22). In the case of gas/solid

catalysis with lipases, several days of continuous reactor operation at temperatures up to 100 °C were achieved (16). Until today, however, only a few examples were known, in which alcohol dehydrogenases were used to catalyze gas-phase reactions (1, 8, 22–25). Besides being labile enzymes, alcohol dehydrogenases require a nicotinamide cofactor NAD(P), which is expensive and sensitive to temperature (26). For economic reasons, the cofactor needs to be regenerated within the system.

In this work, alcohol dehydrogenase from *Lactobacillus brevis* (LBADH), an interesting enzyme with a broad substrate spectrum favoring especially secondary alcohols and ketones (27), was successfully employed to catalyze the reduction of acetophenone to (*R*)-1-phenylethanol in a continuous gas-phase reactor. Regeneration of the cofactor NADP⁺ was performed through the coupled oxidation of 2-propanol to acetone. By the addition of sucrose to the cell extract before immobilization of the enzyme by physical adsorption to glass beads, a tremendous stabilization of the catalyst under reaction conditions was achieved.

Materials and Methods

Chemicals. Acetophenone and ethyl acetate were purchased from Merck (Germany), 2-propanol from Roth (Germany), and sucrose from Aldrich (Germany). The cofactor NADP in both reduced and oxidized form was obtained from Biomol (Germany). All employed chemicals were at least of technical purity grade. Nonporous glass beads of 0.25–0.30 mm diameter were from Braun Biotech International (Germany). Alcohol dehydrogenase from *L. brevis* was supplied as cell extract with a protein content of 9.1 mg/mL and an activity of 1337 μ mol/min/mL (measured photometrically with acetophenone as substrate) as kind gift from Jülich Fine Chemicals

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(Germany). During all experiments, cell extract from the same fermentation batch was utilized.

Enzyme Preparation. In the standard immobilization procedure, 2.5 g of glass beads were added to 1 mL of cell extract containing NADP⁺ in an amount equal to 0.01 g per gram of protein. After 15 min of agitation of the mixture at room temperature, the water was removed under vacuum until dry, free-flowing beads were obtained. To test the influence of sucrose on the stability and activity of the immobilized enzyme, different amounts of sucrose were added to different aliquots of the enzyme/cofactor solution before adding the glass beads (0, 2, and 5 g of sucrose per gram of protein in the cell extract, respectively).

Measurement of Residual Activity of Immobilized Enzyme and Protein Loading of Glass Beads. A 100 mg portion of protein-loaded glass beads was added to 1 mL of deionized water. The resulting mixture was shaken well to release protein from the beads, dissolving it in the water. In preliminary experiments, this procedure allowed complete protein desorption from the glass beads. The enzymatic activity in the solution with the dissolved enzyme was measured using a standard photometric test (27): 10 μ L of this solution was added to a 1 mL cuvette containing 990 μ L of TEA-HCl buffer (pH 7.0) with 11 mM acetophenone and 0.19 mM NADPH. The variation of the NADPH adsorbance at $\lambda = 340$ nm ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) caused by the reaction was followed photometrically (UVIKON 922, Kontron Instruments, UK) for 1 min. The protein content of the solution was measured according to the standard Bradford method (28).

Continuous Gas-Phase Reactions. Continuous gas-phase reactions were performed with a reactor system, similar to a system described elsewhere in the literature (2). The packed bed reactor consisted of a glass tube (20 cm length, 6 mm inner diameter) filled with 0.5 g of protein-loaded glass beads, which were fixed between two plugs of glass wool. Nitrogen, enriched with reactants and with water (by perfusing through substrate and water flasks), was fed into the reactor at a flow rate of 15 mL/min. The reactant concentrations and the water activity in the gaseous reaction mixture entering the packed bed reactor were regulated by a number of flow controllers.

For all experiments, the following conditions were employed: acetophenone activity $a_{\text{AcPh}} = 0.2$, 2-propanol activity $a_{\text{Prop}} = 0.06$, water activity $a_w = 0.5$, total flow rate 15 mL/min. Preliminary experiments showed that at these conditions (i.e., substrate and water activity as well as flow rate) catalyst performance is directly related to the observed substrate conversion. Under such conditions, a decrease in the substrate conversion reveals a deactivation of the catalyst (i.e., either enzyme or cofactor).

The reproducibility of the experiments was verified in preliminary replicate experiments, in which fresh protein-loaded glass beads from the same immobilization batch were used for each run. It was shown that the obtained conversion values vary about 2%.

Gas Chromatographic Analysis. Periodically, 10 mL of the effluent gas was withdrawn from the sampling port of the reactor using a gastight syringe. The reactants were condensed at -18°C and dissolved in 250 μ L of ethyl acetate containing 0.05 mM decane as internal standard. With this method, a complete transfer of the gaseous reactants into the organic solvent was achieved as shown in preliminary tests. If not directly analyzed, the sample solutions were stored at 4°C . For analysis, 1 μ L of this sample was injected (split 1:20) into a Varian

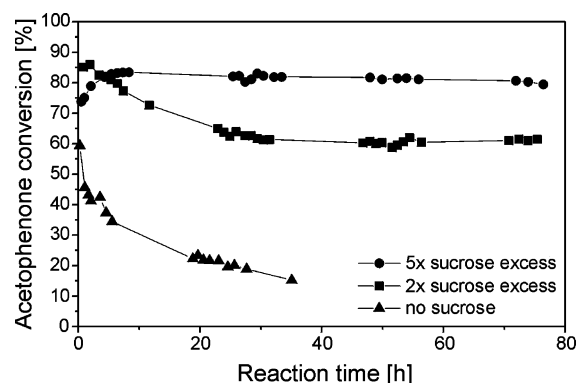


Figure 1. Acetophenone conversion at 40°C for LBADH without sucrose addition, with 2-fold sucrose excess, and with 5-fold sucrose excess. Reaction conditions: $a_w = 0.5$; $a_{\text{AcPh}} = 0.2$; $a_{\text{Prop}} = 0.06$; LBADH loaded beads, 0.5 g; total flow rate, 15 mL/min.

gas chromatograph CP-3800 (USA) equipped with an autosampler (CP 8400, Varian Inc., USA), a flame ionization detector, and a CP-WAX 58 (FFAP) CB (25 m \times 0.25 mm \times 0.2 μ m; Chrompack Inc., USA) column. The injector and detector temperatures were 210 and 280°C , respectively. The column temperature was held at 40°C for 6 min, then increased at $20^\circ\text{C}/\text{min}$ up to 180°C , and kept at this temperature for 4 min. Nitrogen was used as carrier gas with a constant flow of 0.5 mL/min. All samples were analyzed in triplicate. The evaluation of the results was performed with the Star Workstation 5.5 software (Varian Inc., USA) using a five point calibration, acquired each time directly before a new series of samples.

Results and Discussion

Operational Stability of LBADH and NADP. In enzymatic gas/solid systems, the enzyme, being in dry solid form, is normally more stable than in solution (4, 29). Nevertheless, long-term operation of continuous enzymatic gas-phase reactors necessitates a catalyst with extended lifetime. This consequently raises the necessity of an optimization of the enzyme's long-term operational stability, which is especially required for labile enzymes, such as the here-employed alcohol dehydrogenase from *L. brevis*. As can be noted from Figure 1, the stability of the standard LBADH/cofactor preparation at 40°C in gas/solid catalysis is rather limited: the catalyst preparation loses nearly all of its activity during the first day of reaction. A tremendous stabilization of the catalyst preparation, however, was achieved when sucrose had been added during the enzyme immobilization. As can also be seen from Figure 1, increasing amounts of added sucrose managed a progressively better stabilization of the enzyme. By adding a 5-fold excess of sucrose to the enzyme preparation, at 40°C , a nearly complete stabilization of the catalyst was achieved. A half-life of over 40 days (40°C , $a_w = 0.5$) represents a value that raises the stability of LBADH to the level of alcohol dehydrogenases from thermophilic organisms. As a comparison, the thermophilic ADH from *Sulfolobus sulfataricus* (immobilized onto cross-linked albumin glutaraldehyde porous particles) showed a half-life of 19 h at 60°C (8), and another mesophilic alcohol dehydrogenase, the lyophilized ADH from baker's yeast, revealed a half-life of 16 days at 22°C and $a_w = 0.5$ (25).

It is well-known that polyalcoholic substances such as sucrose stabilize solid protein preparations mainly by building a cluster all around the protein and by forming

Table 1. Conversion after 80 h Related to Initial Conversion, Residual Activity of LBADH, and Corresponding Half-Lives Displayed with Continuous Gas-Phase Reactions at 40 °C

sucrose excess in relation to protein content	conversion after 80 h related to initial conversion [%]	residual activity after 80 h (measured in water) [%]	catalyst half-life [d]
no sucrose	25	32	1
2×	71	66	12
5×	96	94	40

hydrogen bonds with the protein surface in place of water. The protein molecules consequently are more compact and thermodynamically stable, and thus they are protected against thermal denaturation (30). Here, it was demonstrated that sucrose is also able to protect the enzyme at higher relative humidity and in the presence of reactants. In addition, the presented results indicate that sucrose present in the catalyst preparation does not negatively influence the catalytic activity of the enzyme.

From Table 1 it can be seen that the conversion after 80 h of reaction (when related to the initial conversion) finds a good correspondence with the residual activity of the LBADH measured in water after this period of time. Thus, the observed activity loss of the catalyst preparation (LBADH and NADP) during gas-phase reaction corresponds to the activity loss of LBADH alone. Thus, the determining step for the loss in conversion observed during the gas-phase reaction is actually the deactivation of LBADH rather than a degradation of NADP. It is conceivable that the cofactor molecules present within the catalyst preparation are complexed by the enzymes and are consequently shielded from external attacks. With the NADP molecules in gas-phase reactions being at least as stable as the LBADH molecules, these findings represent another important advantage of enzymatic gas/solid catalysis compared to enzyme catalysis in liquid phase, where often poor stabilities of nicotinamide cofactors were observed in comparison to the stability of the enzymes molecules (26).

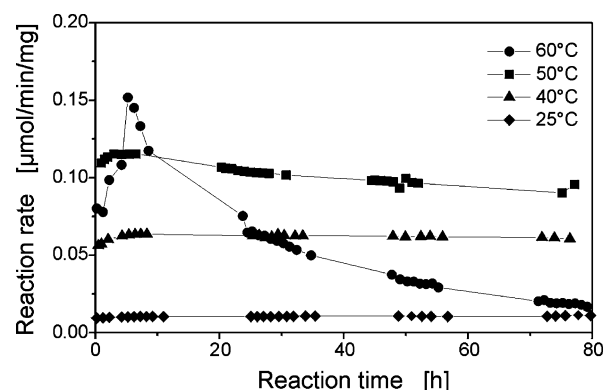
Space–Time Yield of Gas-Phase Reactions Catalyzed by LBADH. The enzyme preparation, optimized in terms of long-term stability, allowed for a first optimization of reaction parameters. Temperature is one of the key parameters in enzymatic gas/solid catalysis, because it has great influence not only on the enzyme activity and stability but also on the reactant concentrations in the gas phase. As can be seen from Figure 2, by raising the temperature from 25 °C to finally 60 °C, an optimum between the reaction rate on the one side and the enzyme stability on the other side can be reached. Up to a temperature of 50 °C, increasing reaction rates are obtained, while the enzyme remains rather stable. At 50 °C, a space–time yield (STY, defined as amount of product synthesized per packed bed volume and per day) of 107 g/L/d was obtained within the first 80 h of reaction (cf. Table 2). At 60 °C, despite the excellent reaction rate at the beginning of the process, the enzyme is degraded very quickly and lower space–time yields are obtained within the considered period of time.

Because in our investigations it was not possible to determine a degradation of the cofactor, the total turnover number (TTN), usually known as number of cycles that can be achieved before the cofactor is destroyed, cannot be calculated as such. Nevertheless, since the loss in conversion observed during the gas-phase reactions was attributed to a degradation of the LBADH, a TTN was calculated with respect to the enzyme. This TTN is defined as amount of product produced per amount of enzyme degraded. From the obtained results, summarized in Table 2, it is obvious that in a continuous gas/

Table 2. Space–Time Yield (STY), Catalyst Total Turnover Number (TTN), and Catalyst Half-Life during Continuous Gas-Phase Reactions Performed at Different Temperatures and $a_w = 0.5^a$

temp [°C]	STY in first 80 h of reaction [g/L/d]	catalyst TTN in first 80 h of reaction	catalyst half-life [d]
25	22	∞	∞
40	66	730,000	40
50	107	260,000	9
60	53	86,000	1

^a The values calculated for space–time yield and catalyst TTN refer to the total reaction time of 80 h.

**Figure 2.** Reaction rate of acetophenone conversion at different temperatures. Reaction conditions: $a_w = 0.5$; $a_{AcPh} = 0.2$; $a_{Prop} = 0.06$; LBADH loaded beads (with 5-fold sucrose excess), 0.5 g; total flow rate, 15 mL/min.

solid system with LBADH producing (*R*)-1-phenylethanol, total turnover numbers can be achieved that are higher than the ones achieved in comparable biotransformations with alcohol dehydrogenases in liquid-phase involving cofactor regeneration. Reduction reactions carried out in a continuous enzyme membrane reactor and catalyzed by (*S*)-ADH from *Rhodococcus erythropolis* showed total turnover numbers up to 1,300 and space–time yields of about 100 g/L/d (31). Optically active propargylic alcohols were obtained by reduction in aqueous solutions with LBADH yielding in a total turnover number of 20,000 (calculated assuming a complete degradation of the cofactor during the batch experiment) (32). Furthermore, the total turnover numbers achieved within the present work are even on the same level as the ones obtained with some of the most successful industrial liquid-phase biotransformations involving cofactor regeneration such as the synthesis of L-leucine (TTN 80,000; STY 214 g/L/d) and L-phenylalanine (TTN 600,000; STY 450 g/L/d) (33).

Conclusions

The alcohol dehydrogenase from *Lactobacillus brevis* was for the first time employed for enzymatic gas/solid catalysis to produce (*R*)-1-phenylethanol. Sucrose, employed during the adsorptive immobilization of enzyme and cofactor, was shown to tremendously increase the operational stability of the catalyst. By addition of sucrose, the half-life of the enzyme during enzymatic gas-

phase catalysis was increased 40 times and the enzyme degradation was irrelevant up to operational temperatures of 50 °C. Thus, now, a simple and efficient method for stabilization of alcohol dehydrogenase and their nicotinamide cofactor for the employment in enzymatic gas/solid catalysis is available.

As a result of the enhanced stability of the catalyst, continuous enzymatic gas-phase reactions could be performed, which showed space–time yields up to 107 g/L/d and catalyst total turnover numbers in the order of 700,000. These results underline that gas-phase reactions with alcohol dehydrogenases including cofactor regeneration can be as effective as the according reactions in liquid environments.

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